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Studies on the Mitochondrially Bound Form of Rat Brain Creatine Kinase

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1. The development of the total rat brain creatine kinase was studied in brain homogenates. Until approx. 14–15 days after birth, the activity remains less than one-third that of the adult activity (207±6 units/g wet wt. S.D.; $n=3$). Over the next 10 days the activity increases markedly to the adult value and thereafter remains essentially constant. 2. In the adult brain, approx. 5% (11.9±2.2 units/g wet wt. S.D.; $n=5$) of the total creatine kinase is associated with the mitochondrial fraction. This creatine kinase could not be solubilized by sodium acetate solutions of up to 0.8M concentration, whereas 66% of the hexokinase associated with brain mitochondria was released under these conditions. 3. Rat brain mitochondria incubated in the presence of various concentrations of creatine (1, 5 and 10mM) and ADP (100μM) synthesized phosphocreatine at rates of approx. 4.5, 11 and 17.5nmol/min per mg of mitochondrial protein. Atractyloside (50μM) or oligomycin (1.5μg/mg of mitochondrial protein) completely inhibited the synthesis of phosphocreatine. 4. The apparent K_m and $V_{max.}$ values of the mitochondrially bound rat brain creatine kinase were determined in both directions. The $V_{max.}$ in the direction of phosphocreatine synthesis is 237nmol/min per mg of mitochondrial protein, with an apparent K_m for creatine of 1.67mM and for $MgATP^{2-}$ of 0.1mM, and in the reverse direction $V_{max.}$ is 489nmol/min per mg of mitochondrial protein, with an apparent K_m for phosphocreatine of 0.4mM and for $MgADP^-$ of 27μM. 5. The results are discussed with reference to the role that the mitochondrially bound creatine kinase may play in the development of brain energy metabolism.

In recent years there have been numerous reports relating the activity of creatine kinase to the control of energy metabolism. Most work has been carried out on heart muscle, where a mitochondrially bound isoenzyme has been detected and proposed to play a central role in intracellular energy transport (Jacobus & Lehninger, 1973; Saks *et al.*, 1974, 1975, 1976). High phosphocreatine concentrations and creatine kinase activities are found also in brain, and Krzanowski & Matschinsky (1969) have proposed phosphocreatine as a possible participant in the control of glycolysis, in addition to being a source of high-energy phosphate. Creatine kinase has also been invoked as functioning in the maintenance of ionic gradients in brain by being coupled with a particularly labile pool of ATP associated with $(Na^+ + K^+)$-stimulated adenosine triphosphatase (Wood & Swanson, 1964; Jongkind, 1974), and in the stimulation of mitochondrial respiration by sustaining local concentrations of ADP (Jacobs *et al.*, 1964; Moore & Jobsis, 1970; Jacobus & Lehninger, 1973; Saks *et al.*, 1975).

Although there have been a number of reports of the association of creatine kinase with brain mitochondria (Jacobus & Lehninger, 1973; Lapin *et al.*, 1974), very little information exist about the activity and metabolic significance of this enzyme associated with relatively pure yet metabolically active rat brain mitochondria. Additionally, information about the development of rat brain creatine kinase, in relation to other mitochondrially bound enzymes, e.g. hexokinase (Land *et al.*, 1977), is also lacking.

The data in the present paper indicate that creatine kinase increases markedly in activity per g wet wt. at a time when greater co-ordination of complex nervous activity is becoming apparent. Further, evidence is provided for the existence of a functionally active mitochondrially bound form of creatine kinase, and the capability of this enzyme to utilize ATP generated from oxidative phosphorylation is demonstrated. The kinetic parameters of the mitochondrial creatine kinase have been investigated and the potential role of this enzyme *in vivo* is discussed.

Experimental

Materials

AMP, ADP, ATP, $NADP^+$, NADH, phosphoenolpyruvate, phosphocreatine, antimycin A, pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC

1.1.1.27), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) hexokinase (EC 2.7.1.1) and creatine kinase (EC 2.7.3.2) were obtained from Boehringer Corp. (London) Ltd. Lewes, E. Sussex BN7 1LG, U.K.). Oligomycin, atractyloside and dithiothreitol were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Bovine plasma albumin (fraction V) was obtained from British Drug Houses, Poole, Dorset BH12 4NN, U.K.

Versilube F50 was a gift from Jacobson, Van den Berg and Co., London W3 7RH, U.K. Pyruvic acid was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and was twice distilled under vacuum and stored at −20°C before use.

Animals

(*a*) *Adults.* Male rats of the Wistar strain (150–250g body wt.) were used throughout. They were fed *ad libitum* on laboratory diet no. 1 (Spratts, Reading, Berks., U.K.), and drinking water and food were always available.

(*b*) *Young animals.* The birth dates of all litters were carefully recorded after inspection, and litters were culled to eight to ten pups. Animals of either sex were used up to weaning (21 days of age). After 21 days of age only male animals were used.

Mitochondrial preparation

Mitochondria were prepared from rat cerebral cortex essentially as described by Clark & Nicklas (1970). The functional integrity of the prepared mitochondria was assessed by measuring their respiration rate and respiratory-control ratios as described previously (Land *et al.*, 1977). In all experiments the mitochondria used had respiratory-control ratios of at least 5 with pyruvate and malate as substrates.

Enzyme assays

These were carried out at 25°C in a Unicam SP.1800 recording spectrophotometer. Creatine kinase was measured by the methods of Saks *et al.* (1975). In the direction of ADP and phosphocreatine synthesis (forward direction), the reaction mixture (volume 1ml) contained the following (final concentrations): 0.25M-sucrose; 20mM-Tris/HCl, pH7.4; 0.8mM-phosphoenolpyruvate; 0.13mM-NADH; 3.3mM-$MgCl_2$; 0.3mM-dithiothreitol; 12 units of lactate dehydrogenase; 6 units of pyruvate kinase; 6μg of oligomycin; 1.5μg of antimycin. Creatine and ATP, the substrates, were added at various concentrations in the determination of the kinetic parameters. The amount of protein included in the assay, either homogenate or mitochondria, was approx. 0.2mg. Triton X-100 (0.1% v/v) was included in the determinations of values for homogenate. Creatine was used to initiate the reactions after a stable baseline A_{340} had been obtained.

The reverse reaction, formation of ATP and creatine, used the following reaction mixture (volume 1ml): 0.25M-sucrose; 20mM-Tris/HCl, pH7.4, 0.6mM-$NADP^+$; 20mM-glucose; 3.3mM-$MgCl_2$; 0.3mM-dithiothreitol; 5.0mM-AMP; 6 units of glucose 6-phosphate dehydrogenase; 6 units of hexokinase. The concentrations of phosphocreatine and ADP were varied in the determination of the kinetic parameters. Approx. 10μg of mitochondrial protein was used in the assays.

As described by Saks *et al.* (1975), when the ATP concentration did not exceed 60% of the Mg^{2+} concentration, all ATP is in the form of $MgATP^{2-}$. Also maximum ADP concentration in the system did not exceed 0.4mM, so that with a Mg^{2+} concentration of 3.3mM the concentration of the $MgADP^-$ complex is equal to $0.9 \times$[ADP].

Hexokinase was assayed by the method of Hernandez & Crane (1966). Creatine was assayed by the method of Bernt *et al.* (1974). ADP was measured by the method of Jaworek *et al.* (1974), and ATP and phosphocreatine were determined essentially by the method of Lamprecht *et al.* (1974), with the modifications that the glucose 6-phosphate dehydrogenase used in the assay was originally freeze-dried and the hexokinase used had been dialysed against 50mM-potassium phosphate buffer, pH7.2. By using these modifications, drift in the assay was considerably diminished. Metabolite measurements were made in a Unicam SP.1800 recording spectrophotometer.

Protein concentration was measured by the biuret method (Gornall *et al.*, 1949).

Mitochondrial incubations

Incubations were carried out at 25°C with constant stirring in an incubation medium containing 100mM-KCl, 75mM-mannitol, 25mM-sucrose, 5mM-pyruvate/Tris, 2.5mM-malate/Tris, 10mM-phosphate/Tris, 10mM-Tris/HCl, 3.3mM-$MgCl_2$, 0.05mM-EDTA, 0.3mM-dithiothreitol, pH7.4 (100mM-K^+ medium). Creatine and inhibitors were added as described in the text. Samples (500μl) were withdrawn at fixed times and the mitochondria separated from their suspending medium by centrifugation at 15000*g* for 2min in an Eppendorf microcentrifuge through 500μl of Versilube F50 silicone oil. The supernatants, which contained negligible creatine kinase activity, were rapidly frozen in liquid N_2, and assayed for phosphocreatine.

Studies on the effect of sodium acetate on the binding of creatine kinase and hexokinase to brain mitochondria

Mitochondria (approx. 2mg of protein) were incubated in sodium acetate solutions (0.1–0.8M)

adjusted to pH 7.4 with Tris base, in a final volume of 500 μl. A sample (200 μl) was taken 10 min after suspension of the mitochondria, and the mitochondria were separated from their suspending medium by centrifugation as above in an Eppendorf bench centrifuge. The supernatant was assayed for creatine kinase and hexokinase activity. An assessment was also made at the end of the incubation of the total creatine kinase and hexokinase activity. That enzyme activity released by treatment of the mitochondria with the various sodium acetate solutions was expressed as a percentage of the total (bound and free) observed activity.

Results

Fig. 1 shows the development of creatine kinase with age, results being expressed as percentages of the adult activity. Creatine kinase activity was measured in the direction of ATP synthesis. The results show a slow increase in the activity of the enzyme until the age of about 15 days (about 40% adult activity), when a sharp increase occurs, which levels off at about 25 days. This activity is thereafter maintained in the adult. In the adult the mitochondrially bound creatine kinase forms only a small percentage of the total activity (approx. 5%), with an activity of 11.9 ± 2.2 μmol/min per g wet wt. ($n=5$) compared with a total tissue activity of 207 ± 6 μmol/min per g wet wt. ($n=3$).

It was not possible therefore to study the development of the mitochondrially bound creatine kinase in the brains of young animals in the same way as has been done for hexokinase (Land *et al.*, 1977), since cytosolic contamination of young brain mitochondria is relatively high compared with that in the adult (Land *et al.*, 1977). However, although in the adult brain only a small percentage of the total creatine kinase is mitochondrially bound, evidence that this fraction is tightly bound to the mitochondria is presented in Fig. 2, where isolated mitochondria have been suspended in sodium acetate solutions of increasing ionic strength, and the solubilization of creatine kinase and hexokinase has been monitored. Although mitochondrially bound hexokinase shows an increase in percentage release from approx. 20% in 0.1 M-sodium acetate to about 70% at 0.6 M-sodium acetate, creatine kinase maintains a basal value of approx. 15% release up to 0.8 M-sodium acetate.

This suggests that the interaction between the brain mitochondrial membrane and creatine kinase is considerably stronger than that with hexokinase, since 70% of the hexokinase bound to adult brain mitochondria may be released by an ionic strength of 0.6*I* (Wilson, 1968; Land *et al.*, 1977), whereas the creatine kinase remains tightly bound even after 10 min incubations in a medium of ionic strength 0.8*I*.

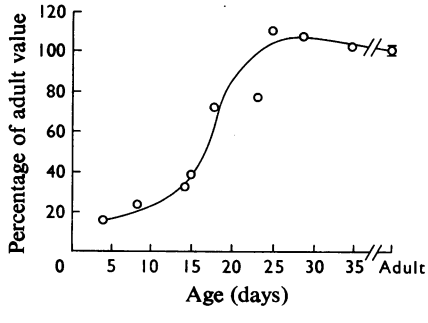

Fig. 1. *Development of creatine kinase in rat brain*
Creatine kinase activities were measured in cerebral-cortex homogenates of rats of different ages as outlined in the Experimental section. Results are expressed as percentages of the adult value (207 ± 6 units/g wet wt.). Each point represents the mean $\pm$S.D. obtained from a minimum of three distinct determinations of enzyme activity, each of which was performed in triplicate on a pooled homogenate preparation derived from four animals of the same age.

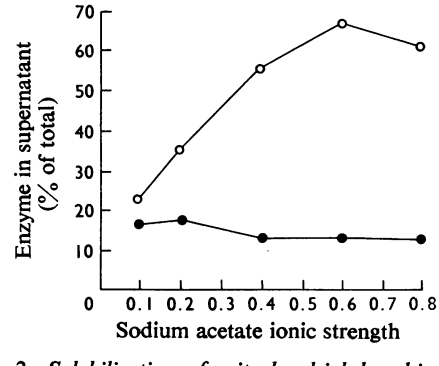

Fig. 2. *Solubilization of mitochondrial hexokinase and creatine kinase by sodium acetate*
Mitochondria from adult brains were prepared as outlined in the Experimental section and then incubated at 25°C with stirring in sodium acetate solutions of various ionic strengths. Samples were taken at 10 min and assayed for total and free hexokinase (○) and creatine kinase (●), as described in the Experimental section. The results are expressed as the amount of enzyme in the supernatant expressed as percentages of the total (bound+free) enzyme present.

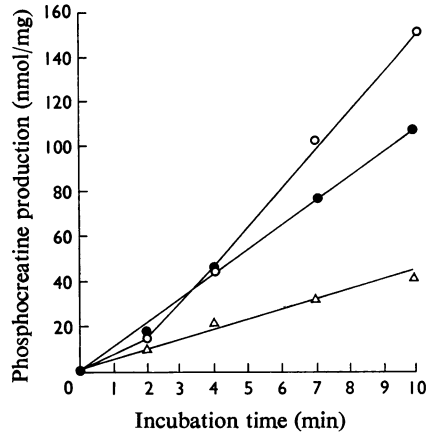

Fig. 3. *Phosphocreatine production by creatine kinase bound to rat brain mitochondria*

Mitochondria from adult rat brains were prepared as outlined in the Experimental section. These were incubated at a concentration of 1.32mg/ml in a final volume of 3ml in the medium outlined in the Experimental section for mitochondrial incubations with the system being oxygenated. Samples were withdrawn at fixed time-intervals and assayed for phosphocreatine as described in the Experimental section. The reaction was initiated by the addition of 300nmol of ADP at zero time. The rates of production of phosphocreatine are shown for 10mM- (○), 5mM- (●) and 1mM- (△) creatine.

Fig. 3 shows the generation of phosphocreatine by brain mitochondria from added creatine and ADP under State-3 (Chance & Williams, 1956) oxidative conditions. It is clear that the rate of phosphocreatine formation is proportional to the creatine concentration and linear for the period of the experiment (10min), except for a slight lag at the beginning, with the high creatine concentration (10mM). The rates of phosphocreatine formation were 4.5, 11 and 17.5nmol/min per mg of mitochondrial protein in the presence of 1mM-, 5 mM- and 10mM-creatine respectively.

Presumably the ADP added is being phosphorylated by the electron-transport chain to ATP, which is then phosphorylating the creatine. Since only 300nmol of ADP was added at zero time, and after 10min with 10mM-creatine in excess 600nmol of phosphocreatine has been generated, it is clear that the ADP is recycling for further ATP formation after the synthesis of phosphocreatine.

Atractyloside (50μM), which inhibits transfer of matrix ATP into the extramitochondrial space, completely inhibits the formation of phosphocreatine during these experiments, showing the dependence of synthesis on the oxidative phosphorylation of ADP in the mitochondrial matrix and confirming that mitochondrial creatine kinase lies on the cytosol side of the atractyloside-sensitive barrier, as suggested by Craven *et al.* (1969). The dependence on oxidative phosphorylation was confirmed by the complete

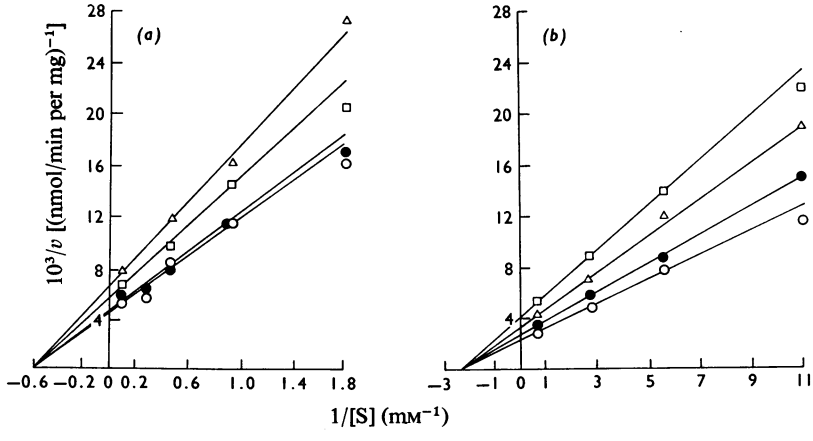

Fig. 4. *Kinetics of creatine kinase bound to rat brain mitochondria*

Mitochondria from adult rat brains were prepared as outlined in the Experimental section. Creatine kinase activity was determined at various creatine and $MgATP^{2-}$ concentrations (Fig. 4*a*) and in reverse at various phosphocreatine and $MgADP^-$ concentrations (Fig. 4*b*). The results are plotted as Lineweaver–Burk plots and the derived $V_{max.}$ rates and apparent Michaelis constants (K_m) are presented in Table 1. (*a*) Creatine [S] was varied between 0 and 11mM at four different $MgATP^{2-}$ concentrations (○, 2mM; ●, 1mM; □, 0.3mM, △, 0.2mM). (*b*) Phosphocreatine [S] was varied between 0 and 2mM at four different $MgADP^-$ concentrations (○, 420μM; ●, 84μM; △, 42μM; □, 25μM).

Table 1. *Kinetic parameters of the mitochondrially bound form of rat brain creatine kinase*

Substrate	K_m (mM)
$MgADP^-$	0.027
Phosphocreatine	0.41
$MgATP^{2-}$	0.104
Creatine	1.67

$V_{max.}$ for ATP+creatine $\rightleftharpoons$ ADP+phosphocreatine = 237 nmol/min per mg of mitochondrial protein

$V_{max.}$ for ADP+phosphocreatine $\rightleftharpoons$ ATP+creatine = 489 nmol/min per mg of mitochondrial protein

inhibition of phosphocreatine synthesis by the addition of oligomycin (2 μg/ml).

The kinetics of mitochondrial creatine kinase were investigated under conditions where the initial concentrations of substrates were varied and the products kept near zero concentrations by using a trapping system as suggested by Saks *et al.* (1975). The activity of brain mitochondrial creatine kinase was measured at four different concentrations of $MgATP^{2-}$, and the creatine concentration was varied at each $MgATP^{2-}$ concentration (Fig. 4*a*). Likewise in the reverse direction, the enzyme activity was measured at four different $MgADP^-$ concentrations, the phosphocreatine being varied at each concentration (Fig. 4*b*). In both cases, the Lineweaver–Burk plots intersect at the same point on the abscissa, supporting, under these conditions, a mechanism of independent binding of nucleotides and guanidine substrates [cf. Saks *et al.* (1975) for heart mitochondrial isoenzyme]. The derived $V_{max.}$ and K_m values for the brain mitochondrial creatine kinase are presented in Table 1.

Discussion

The developmental pattern of creatine kinase indicates a rapid increase in the overall activity of creatine kinase at the age of 15–20 days in the young rat. This corresponds with the time at which rapid neuronal growth and establishment of synapses is occurring (McIlwain & Bachelard, 1971) and is shortly after the time of development of the tricarboxylic acid-cycle enzymes (e.g. citrate synthase) but approximately coincident with the development of the brain pyruvate dehydrogenase and mitochondrially bound hexokinase activity (Land *et al.*, 1977). The increase in enzyme activity therefore corresponds to the time at which a rapid increase in the co-ordinated neuronal activity with its concomitant energy requirement will be occurring. Lapin *et al.* (1974) have also observed a similar marked increase in brain creatine kinase activity at a corresponding developmental period in mouse brain. In view of the proposed relationship between creatine kinase and energy homoeostasis (Krzanowski & Matschinsky, 1969; Vial *et al.*, 1972; Saks *et al.*, 1975), it may be that the increase in creatine kinase activity plays an integral part in the maintenance of brain energy metabolism.

The ability to solubilize hexokinase to a large extent by suspension in a high-ionic-strength medium suggests a weak interaction between the hexokinase and the mitochondrial membrane. Specific solubilization of mitochondrial hexokinase has been demonstrated with glucose 6-phosphate (Land *et al.*, 1977). The inability of a medium of equal high ionic strength to solubilize the creatine kinase associated with brain mitochondria would indicate a relatively strong interaction between the enzyme and the mitochondrial membrane and may lend support to the original proposal by Jacobs *et al.* (1964) that the mitochondrial and cytosolic forms of creatine kinase are different isoenzymes, a situation that is paralleled in the heart (Saks *et al.*, 1974).

The ability of brain mitochondrial creatine kinase to synthesize phosphocreatine from creatine and ATP generated from oxidative phosphorylation could be of some significance to the brain by the consequent maintenance of relatively high ADP concentration in the immediate vicinity of the mitochondria, thereby ensuring continued respiration. This role has been suggested for mitochondrially bound hexokinase in brain (Moore & Jobsis, 1970; Land *et al*, 1977) and creatine kinase in heart (Jacobus & Lehninger, 1973; Saks *et al.*, 1974), and may now be extended to creatine kinase associated with brain mitochondria. The concentrations *in vivo* of creatine (5.12 μmol/g wet wt.; approx. 5 mM) and ATP (2.45 μmol/g wet wt.; approx. 2.5 mM) (Veech *et al.*, 1973) are sufficiently high to suggest that phosphocreatine synthesis may occur *in vivo*, since the K_m values of the creatine kinase for creatine and $MgATP^{2-}$ are 1.67 mM and 104 μM respectively (Table 1). However, the high affinity of creatine kinase for $MgADP^-$ and phosphocreatine (Table 1), together with the relatively high rate of the reverse reaction, suggest that, as in heart, for synthesis of phosphocreatine in net terms *in vivo*, some control of the reverse reaction may be necessary. This may be, as for the heart isoenzyme, the concentration of free Mg^{2+} (Saks *et al.*, 1975). Provided that the Mg^{2+} concentration is low, only $MgATP^{2-}$ will exist, since the stability constant for $MgATP^{2-}$ is high compared with that of $MgADP^-$ (Alberty, 1968). As the Mg^{2+} concentration is raised, more $MgADP^-$ will be formed, thus allowing the reverse reaction to occur.

The experiments reported here suggest that the creatine kinase is located on the outside of the atractyloside-sensitive barrier of the mitochondrial

membrane, which may or may not be the outer mitochondrial membrane, as suggested by Craven *et al.* (1969). Further, the complete inhibition of phosphocreatine synthesis brought about by the addition of either atractyloside or oligomycin demonstrates the dependence of phosphocreatine formation on the generation of ATP by oxidative phosphorylation and its subsequent transport to the site of the enzyme.

Clearly a mitochondrially bound form of creatine kinase exists in brain (the present paper), heart (Jacobus & Lehninger, 1973; Saks *et al.*, 1975) and skeletal muscle (Jacobs *et al.*, 1964), all of which are tissues subject to bioelectric excitability. Although in the adult brain only about 5% of the total brain creatine kinase activity (approx. 10 μmol/min per g wet wt.) is associated with the mitochondria, this activity is of the same order as that of the mitochondrially bound brain hexokinase (approx. 9 μmol/min per g wet wt.; Land *et al.*, 1977). Hence it is clear that in brain both kinases will be competing for the available ATP, particularly since their K_m values for ATP are of the same order [for $MgATP^{2-}$: creatine kinase, 104 μM (Table 1); hexokinase, 376 μM (R. F. G. Booth, unpublished work).] It may be argued that the association of both hexokinase and creatine kinase with the mitochondrial membrane may give these enzymes a 'privileged access' to ATP from oxidative phosphorylation compared with other ATP-requiring processes. The need for this in the case of hexokinase may be rationalized on the basis of a need for a continuous and highly active glycolytic flux in the fully developed and responding brain (see Land *et al.*, 1977). The similar developmental pattern of creatine kinase to that of hexokinase suggests that creatine kinase may also be involved in the overall co-ordination of energy metabolism and neurotransmission in the fully active adult brain. The mitochondrially bound creatine kinase would be preferentially placed to channel ATP from oxidative phosphorylation into phosphocreatine in the cytosol. This phosphocreatine could then be used by the highly active cytosolic creatine kinase for regeneration of ATP concentrations at localized sites of energy demand, e.g. membrane-bound $(Na^+ + K^+)$-stimulated adenosine triphosphatase. The use of phosphocreatine in this way as an energy pool would provide several advantages to the brain. It would act as an energy buffer so that during repolarization of the nerve membrane the steady-state ATP concentration would be maintained essentially constant; this would be of considerable advantage to the brain, because it allows the channelling of energy to those processes requiring it without perturbing in any marked way the various ATP pools, which would have considerable repercussions for other ATP-requiring reactions and also for the cellular free Mg^{2+} concentration. Secondly, phosphocreatine could act as a pool for P_i; thus under conditions where phosphocreatine was utilized the steady-state concentration of P_i would rise with its consequent influence on oxidative phosphorylation via the phosphate potential ($[ATP]/[ADP][P_i]$) (Wilson *et al.*, 1974). Lastly, phosphocreatine has been implicated as a possible inhibitor of phosphofructokinase (Krzanowski & Matschinsky, 1969), thus acting as a regulator of glycolysis. It may be, therefore, that the coincidence of the development of the creatine kinase and hexokinase in rat brain and also of their association with the brain mitochondrial membrane is an adaptation by the brain and possibly by other tissues involved in bioelectric phenomena, to permit the maintenance of a highly efficient and organized energy production and transfer system, necessary to their specialized activities.

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References

Alberty, R. A. (1968) *J. Biol. Chem.* **243**, 1337–1343

Bernt, E., Bergmeyer, H. U., Mollering, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 1772–1776, Verlag Chemie, Academic Press, New York and London

Chance, B. & Williams, G. R. (1956) *Adv. Enzymol.* **17**, 65–134

Clark, J. B. & Nicklas, W. J. (1970) *J. Biol. Chem.* **245**, 4724–4731

Craven, P. A., Goldblatt, P. J. & Basford, R. E. (1969) *Biochemistry* **8**, 3525–3532

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Hernandez, A. & Crane, R. K. (1966) *Arch. Biochem. Biophys.* **113**, 223–229

Jacobs, H., Heldt, H. W. & Klingenberg, M. (1964) *Biochem. Biophys. Res. Commun.* **16**, 516–521

Jacobus, W. E. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248**, 4803–4810

Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2127–2129, Verlag Chemie, Academic Press, New York and London

Jongkind, J. F. (1974) in *Recent Advances in Quantitative Histo-and Cyto-Chemistry* (Dubach, V. C. & Shimdt, V., eds.), pp. 210–227, Hans Huber, Berne

Krzanowski, J. & Matschinsky, F. M. (1969) *Biochem. Biophys. Res. Commun.* **34**, 816–823

Lamprecht, W., Stein, P., Heinz, F. & Weisser, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 1777–1779, Verlag Chemie, Academic press, New York and London

Land, J. M., Booth, R. F. G., Berger, R. & Clark, J. B. (1977) *Biochem. J.* **164**, 339–348

Lapin, E, P., Maker, H. S. & Lehrer, G. M. (1974) *J. Neurochem.* **23**, 465–469

McIlwain, H. & Bachelard, H. S. (1971) *Biochemistry and the C.N.S.*, 4th edn., Churchill-Livingstone, London

Moore, C. L. & Jobsis, F. F. (1970) *Arch. Biochem. Biophys.* **138**, 295–305

Saks, V. A., Chernousova, G. B., Voronkov, Yu. I., Smirnov, V. N. & Chazov, E. I. (1974) *Circ. Res.* **35**, *Suppl.* **3**, 138–149

Saks, V. A., Chernousova, G. B., Gukovsky, D. E., Smirnov, V. N. & Chazov, E. I. (1975) *Eur. J. Biochem.* **57**, 273–290

Saks, V. A., Chernousova, G. B., Vetter, R., Smirnov, V. N. & Chazov, E. I. (1976) *FEBS Lett.* **62**, 293–296

Veech, R. C., Harris, R. C., Veloso, D. & Veech, E. H. (1973) *J. Neurochem.* **20**, 183–188

Vial, C., Godinot, C. & Gautheron, D. (1972) *Biochimie* **54**, 843–852

Wilson, J. E. (1968) *J. Biol. Chem.* **243**, 3640–3647

Wilson, D. F., Erecinska, M. & Dutton, P. L. (1974) *Annu. Rev. Biophys. Bioeng.* **3**, 203–230

Wood, T. & Swanson, P. D. (1964) *J. Neurochem.* **11**, 301–307